Copper I transfer between *Enterococcus hirae* CopA, CopZ and CopY *in vitro*

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Binding and transfer of Cu(I) between three key proteins in the bacterium *Enterococcus hirae* has been studied. CopA, an ATPase protein, was truncated, and its metal binding domain (AMBD) was expressed as a fusion with maltose binding protein (abbreviated as AMBD-MBP). AMBD-MBP will bind and transfer 1 mol equivalent of Cu(I) to the repressor protein CopY *in vitro*. AMBD, which also has a higher relative affinity for Cu(I) than CopZ, can also perform the same Cu(I) transfer role *in vitro* that CopZ does. The ability of the metal binding domain of CopA to transfer Cu(I) to CopY suggest it may serve that role in *E. hirae* and possibly in those organisms that don't possess a CopZ type copper chaperone.

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Introduction

Copper (Cu) is an essential element to all organisms because of its use as a cofactor in enzymes that catalyze the transfer of electrons [1]. Copper is utilized in electron transfer processes because of its ability to cycle between the oxidation states of Cu(I) and Cu(II) [2]. The cycling between oxidation states makes copper ions toxic to both euraryotic and prokaryotic cells because of their ability to support oxidative damage to proteins, nucleic acids, and lipids [1]. Therefore, the regulation of the intercellular levels and location of copper ions is crucial to cellular homeostasis [3].

Cu(I) ATPase proteins are conserved from bacteria to humans and typically serve as an import/export Cu(I) pump. Cu(I) chaperones, another class of Cu(I) proteins, exist in most systems and are proposed to work together with the Cu(I) ATPases to route Cu(I) within the cell. For example, *Escherichia coli* (*E. coli)* and *Mycobacterium tuberculosis (M. tuberculosis)* possess ATPase proteins which perform bidirectional routing of Cu(I) into and out of the cell, acting as both an import/export Cu(I) pump [4, 5].

The bacterium *Enterococcus hirae* (*E. hirae*) is one of the simplest and best understood Cu(I) systems [6-8]. Within this system the ATPase CopA is believed to import Cu(I) into the cell. The intracellular metal binding domain (MBD) of CopA is proposed to accept Cu(I) from the ATPase portion of the protein and transfer the copper to the chaperone CopZ. The metal binding domain of CopA is highly homologous to CopZ. In conditions of excess copper CopZ shuttles the Cu(I) to the DNA bound repressor protein Zn(II) CopY, which has a higher affinity for Cu(I) than Zn(II), displacing the Zn(II) and binding the Cu(I). *E. hirae* also contains another ATPase protein, CopB, which is proposed to export Cu(I) out of the cell [9].

The role of the cytosolic metal binding domain of CopA (AMBD) is not well defined. Homology of the MBD to CopZ, and the cytosolic metal binding domains of the Menkes and Wilsons

ATPases, strongly suggest it will bind Cu(I) at a stoichiometry of 1:1; but it is not clear from where it can acquire the metal or whether it can supply it other proteins as the copper chaperones do. Following confirmation of its ability to bind the copper (I) ion, the CopA metal binding domain, AMBD, its ability to transfer Cu(I) to CopA and CopY was analyzed through direct transfer and spectroscopic analyses.

Materials and Methods

1. Metal Titrations.

Cu(I) titrations were prepared as Cu(I) acetonitrile (ACN) perchlorate (Cu (I) $(CH_3CN)_4ClO_4$) salt dissolved in 60% acetonitrile [10]. The solid Cu(I) compound was reconstituted into solution in an anaerobic Omni-Lab glove box, Vacuum Atmosphere Company. Concentrations of the Cu(I) stock solutions were determined by flame atomic absorption spectroscopy (FAAS). Cu(I) was added to 5 nmol of protein in 2.5 nmol increments or 0.5 molar equivalents into a final volume of 1.5 mL. All titrations were performed in the glove box and samples were transported in anaerobically sealed screw top cuvettes (Spectrocell, Inc., Oreland, PA, USA) for spectral analysis outside of the glove box.

2. Spectral Analyses.

All UV-visible spectroscopy was performed on a Cary 1E UV-Visible Spectrophometer at 22°C. Titrations were followed by measuring the formation of a Cu(I)-S ligand to metal charge transfer band (LMCT) at 250 nm in the absorption spectrum between 200-420 nm [11]. Luminescence measurements of the solvent shielded Cu(I)-thiolates in Cu(I) CopY were performed using a Perkin Elmer LS50B Luminescence Spectrometer (Perkin Elmer, Waltham, MA, USA) [12]. Luminescence spectra in the range of 500-600 nm were recorded upon excitation by 295 nm light [11, 12]. A 350 nm band pass filter was used to remove second order effects and excitation and emission slit widths were set at 5 and 20 nm, respectively. Flame Atomic Absorption Spectroscopy was carried out on a Perkin-Elmer 1100 Spectrophotometer (Perkin Elmer, Waltham, MA, USA).

3. Plasmid Preparation.

Plasmid, pHT1, containing CopA DNA was prepared via alkaline lysis followed by a polyethylene glycol (PEG). The plasmid was then digested with restriction enzymes BbvCI and XhoI to eliminate the unwanted membrane portion of the gene [13, 14]. The DNA was incubated with Klenow fragments in order to create blunt ends and then resolved on an agarose 1.5% analytical gel. Two fragments were produced: 4.9kb and 1.9kb. The 4.9kb fragment was excised from the gel and the StrataPrep (Stratagene, La Jolla, CA, USA) kit was used to purify the DNA. The ends were then self-ligated and transformed into XL-1 blue supercompetent cells (Stratagene, La Jolla, CA, USA). AMBD, the fragment containing the metal binding domain of CopA, was then cloned onto the NEB (New England Biolabs, Ipswich, MA, USA) pMAL-c2X vector by the ligation at BamHI and SalI restriction enzymes sites which were created by PCR based site-directed mutagenesis. The resulting plasmid, pAMBD-MBPmbd, encoded the cytoplasmic portion of the CopA protein fused to the maltose binding protein (AMBD-MBP). pAMBD-MBPmbd in addition to containing the protein fusion construct also contains a N-terminus His-tag and a factor X cleavage site (see supplemental information for plasmid map).

4. Protein Purification.

CopZ and CopY were purified to homogeneity by following the previously published methods [12, 15]. Large (4-6 L) of cell cultures containing BL21 (DE3) *E. coli* (Novagen, Madison, WI, USA) transformed with the pAMBD-MBPmbd plasmid were grown at 37°C to an OD_{600} of 0.6-1.00 in LB media containing 200 μg/ml ampicillin + 1 gram dextrose/500 ml culture. Protein expression was induced at an OD_{600} of 1, with 0.3 mM IPTG and the cells were incubated for an additional two hours. The cells were harvested by centri-

fugation at 5,000 x g for 20 minutes and the supernatant was discarded. The cell pellet was resuspended in 50 ml of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) and stored at -20°C over-night. The cell pellet was thawed in cold water and lysed by sonication. The lysate was then centrifuged at 9,000 x g in a Sorvall SS-34 rotor for 30 minutes at 4°C. Solid enzyme grade ammonium sulfate was added slowly to the crude supernatant to reach a 45% of saturation. After a 30 minute incubation in an ice-water bath, the sample was centrifuged 5,000 x g in a Sorvall SS-34 rotor. The supernatant was recovered and diluted 1:1 with deionized water. The AMBD-MBP sample was then loaded onto a column containing a 2 ml NEB Amylose resin (New England Biolabs, Ipswich, MA, USA) equilibrated with 20 mM Tris-Cl (pH 7.4), 10 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol (column buffer). After loading the sample, the column was washed with column buffer until A_{280} < 0.05, followed by protein elution of the AMBD-MBP with a 0-100% gradient over 60 ml of column buffer + 10 mM maltose. The fractions containing AMBD-MBP were pooled and loaded onto a DEAE Fractogel® (Merck Chemicals Ltd., Nottingham, United Kingdom) column run at 4°C and equilibrated with column buffer. The column was washed with this buffer until A_{280} < 0.05. AMBD-MBP was eluted with a 0-0.1 M NaCl gradient over 680 ml total volume. Fractions containing AMBD-MBP were pooled, concentrated in an ultrafiltration device (Amicon) (EMD Millipore Corporation, Billerica, MA, USA) fitted with a 30,000 (YM30) nominal molecular weight cut-off membrane and then reduced. Reduction of AMBD-MBP was achieved with a mixture of 6M guandidine-HCl, 100 mM EDTA and 150 mM DTT in accordance with the procedure previously described for metallothionein [16]. After a two-hour incubation at 42°C, the sample was transferred into the Omni-Lab anaerobic glove box, and resolved on a Sephadex G-25 column equilibrated in 25 mM HCl at room temperature to remove the excess DTT, DTT-DTT, and salts. One ml of 1M HCl was loaded before and after protein loading. Reduction of the protein was verified by the DTDP assay [17]. Purity of the protein was determined by SDS-PAGE.

5. Ultrafiltration Experiment Protocol.

An ultrafiltration microcentrifuge assay was developed to address the transfer of copper between the cupro-proteins - AMBD, CopY and CopZ. The assay tests the ability of proteins to exchange copper with one another; following the exchange process the proteins are separated by ultrafiltration. The separation device consisted of a Microcon YM30 (EMD Millipore Corporation, Billerica, MA, USA), a microcentrifuge tube equipped with a cup insert with an Amicon 30,000 nominal molecular weight cutoff membrane bottom, with an aqueous sample of Zn(II) CopY in the micro centrifuge tube (figure 1). Small proteins, Cu(I)CopZ and Cu(I)-AMBD for example, and Cu-complexes can penetrate the cup membrane while larger proteins are retained above the membrane. AMBD-MBP is 45,000 Daltons and is retained above the membrane, CopZ is 11,000 Daltons, by itself should flow through the membrane, and AMBD is 14,000 Daltons, by itself should flow through the membrane and any trace Cu(I) can flow through the membrane. Cu(I) that passes through the membrane and presented to Zn(II)CopY in the right form is detected through the Cu(I) specific luminescence of Cu(I)CopY [11]. The reactions, both test and control, are shown in table 1.

All of the samples were centrifuged at 2,100 x g for 20 minutes in an anaerobic environment and then the final mixture contained at the bottom of the centrifuge tube was analyzed using luminescence spectroscopy.

Results

1. Cu(I) titration into the AMBD-MBP fusion.

To determine stoichiometry of metal binding in AMBD, Cu(I)ACN was titrated into the purified AMBD-MBP fusion protein. The stoichiometry

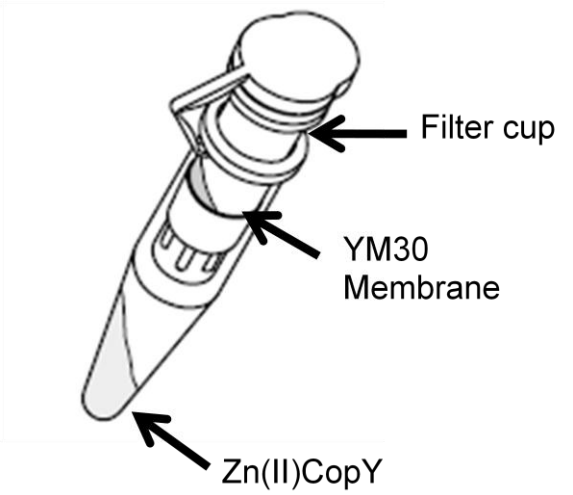

Figure 1. Ultrafiltration schematic**:** in each experiment Zn(II)CopY is place in the bottom of the centrifuge tube. Protein transfer reactions and cleavage of constructs with Factor X are carried out in the filter cup. After a brief incubation the tubes are centrifuged at 10,000 x g to separate the components and enable potential Cu(I) carriers to mix with Zn(II)CopY.

of Cu(I) binding to proteins can be monitored by the increase in absorbance at approximately 250 nm [11]. The absorbance increase is due to the formation of a ligand-to-metal charge transfer band (LMCT) between Cu(I) and the two cysteinyl sulfurs found in AMBD. Maltose Binding Protein (MBP) does not contain cysteine residues [11]. The addition of increasing amounts of Cu(I) increases the LMCT band until the metal binding site becomes saturated with Cu(I). Additional Cu(I) does not bind to the protein and the measured absorbance reaches a plateau. The Cu(I) titration into AMBD-MBP, as measured by UV-Visible spectroscopy, indicates a stoichiometry of one mole of Cu(I) per one mole of AMBD-MBP (figure 2). Luminescence characteristic of Cu(I) going into a solvent shielded sight can be monitored with excitation at 295 nm between 500 and 700 nm. The Cu(I) chelated by AMBD-MBP is in an exposed site so it does not luminesce.

2. Cu(I) transfer from AMBD-MBP fusion to CopY.

The Cu(I)-loaded metal binding domain of CopA fused to maltose binding protein (AMBD-MBP) was titrated directly into Zn(II)CopY to determine if Zn(II)CopY can accept Cu(I) from AMBD-MBP. The increasing addition of Cu(I) into the solvent-shielded environment of CopY monitored through luminescence assays [11]. The fusion protein containing the metal binding domain of CopA and Maltose Binding Protein (AMBD-MBP) which has previously shown to bind one mole of Cu(I) per mole of AMBD-MBP, was added to Zn(II)CopY in increasing amounts while luminescence was monitored. The measurement of luminescence arising from the formation of Cu(I)-S bonds in the solvent-shielded metal binding site of CopY was observed with the addition of increasing amounts of Cu(I) from AMBD-MBP to Zn(II)CopY. The luminescence increased at 600 nm until a plateau was reached at a ratio of two moles of Cu(I) per mole of CopY (figure 3). These data are consistent with the Cu(I) titrations of CopY seen in previous studies [11, 12].

3. Cu(I) transfer specificity AMBD-MBP to CopZ.

Within *E. hirae,* the ability of CopZ to obtain Cu(I) from Cu(I)AMBD-MBP and ability to transfer the Cu(I) to CopY is unknown. AMBD-MBP's ability to acquire Cu(I) from Cu(I)CopZ is also unknown. CopZ could compete with the metal binding domain of CopA for the Cu(I) transfer or support the transfer since the metal binding domain of CopA is fixed to the membrane. The ability of the metal binding domain of CopA (AMBD) to deliver Cu(I) to CopY was determined when AMBD was cleaved from maltose binding protein (MBP) with Factor X.

4. Ultrafiltration experiment.

Copper(I) transfer between AMBD-MBP, AMBD, CopZ and subsequently to CopY was measured with the ultrafiltration assay (figure 1). The luminescence assay results from each set of experiments are provided in table 1. In the first reaction, reaction 1, Cu(I)CopZ was incubated over the membrane by itself, following the centrifugation it was determined that it had passed its copper to CopY. Cu(I)CopZ has previously been shown by several methods to be able to transfer Cu(I) to CopY [11, 12]. To

Table 1: Ultrafiltration Micro-Centrifuge Experiment: 10 separate experiments were performed. Each of the samples mentioned above are summarized in the table. A "yes" for luminescence indicated that a luminescence spectra was produced at 600nm with excitation at 295nm within the range of 500-700nm, indicating a Cu(I) transfer between the sample and CopY. A "no" for luminescence indicated that no spectra were produced within the same range, indicating no Cu(I) transfer.

Reaction	Sample and sample modifiers	Cu(I)Transfer (YES/NO)
1	Cu(I)CopZ	**YES**
2	S-tagCu(I)CopZ + S-tag Resin*	NO
3	Cu(I)AMBD-MBP	NO
4	Cu(I)AMBD-MBP, apo-CopZ	NO
5	Cu(I)AMBD-MBP, Factor X**	**YES**
6	Cu(I)AMBD-MBP, Factor X**, His-Select^{++}	NO
7	Cu(I)AMBD-MBP, Factor X**, His-Select^{++}, apo-CopZ	NO
8	AMBD-MBP, Factor X, His-Select^{++}, Cu(I)CopZ	NO
9	S-tagCu(I)CopZ + S-tag Resin*, apo-CopZ	**YES**
10	Cu(I)AMBD-MBP, glutathione	NO

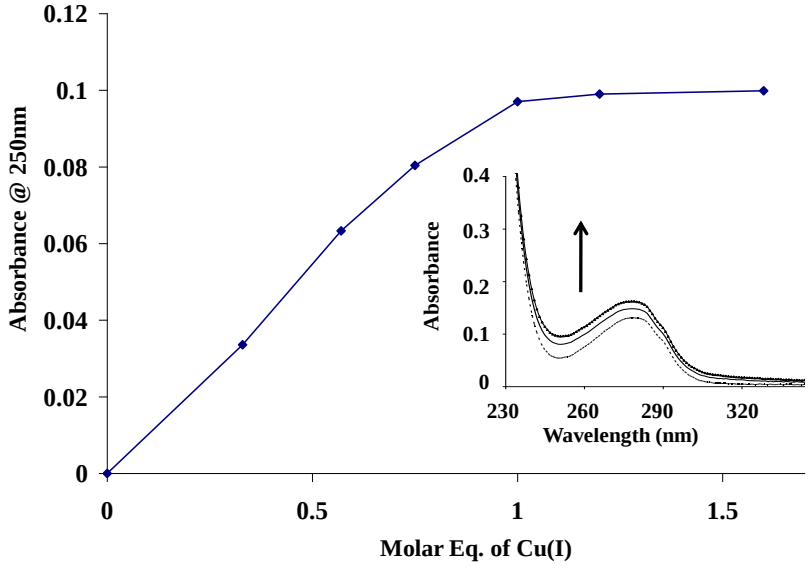

Figure 2. UV-visible Spectral analysis of Cu(I) titration to AMBD-MBP. **Main figure:** Molar Eq. of Cu(I) were added as a function of the increasing LMCT to a plateau of maximum copper binding. In AMBD-MBP, the maximum copper binding is reached at approximately 1.0 molar equivalents of Cu(I) ACN based on thiolate content. **Inset**: The UV-visible spectra of AMBD-MBP titrated with increasing amounts of Cu(I)ACN. Cu(I)ACN was titrated into 5 nmol of *apo*- AMBD-MBP in approximately 0.25 molar Eq. The increasing concentration of Cu(I)ACN yields an increasing Cu(I)-S (LMCT) band near 250nm. The initial absorbance is due to the aromatic residues found within AMBD-MBP.

insure that the Cu(I)CopZ transfer was dependent on the protein passing through the membrane and not merely the release of Cu(I) another sample of Cu(I)CopZ was attached to the S-tag affinity resin, reaction 2. CopY can chelate "free" Cu(I) and small Cu(I)-complexes like the Cu(I)-acetonitrile. Cu(I)CopZ attached to the resin did not transfer its Cu(I). The large

Cu(I)AMBD-MBP complex, reaction 3, was unable to transfer its Cu(I) to CopY. Incubation of Cu(I)AMBD-MBP with apo-CopZ was similarly unable to transfer Cu(I) to CopY, reaction 4. The *E. hirae* model described above proposes that CopA imports Cu(I) into the cell so it was rational to determine whether CopZ might accept Cu(I) from the metal binding domain, AMBD. Intriguingly, reaction 5, incubation of Cu(I)AMBD-MBP with the Factor X protease which cleaves the AMBD from the MBP does enable Cu(I) to be passed to CopY. To insure that the cleavage reaction was not directly enabling the Cu(I) to be released from the AMBD the cleaved AMBD was incubated with His-Select resin (Sigma-Aldrich, St. Louis, MO, USA). Cu(I)AMBD bound to the His-Select resin does not transfer Cu(I), reaction 6. Incubation of apo-CopZ with the His-Select bound AMBD did not result in Cu(I) transfer, reaction 7. On the other hand, the Cu(I) transfer effected by Cu(I)CopZ, reaction 1, is blocked when it is incubated with apo-AMBD attached to the His-Select resin, reaction 8. It is presumed the AMBD removed the Cu(I) from Cu(I)CopZ. Apo-CopZ can remove Cu(I) from Cu(I)CopZ linded to the S-tag resin, demonstrating that the inability to remove Cu(I) from the 2 the 3 forms of AMBD lies not with the apo-CopZ, reaction 9. Lastly, glutathione, a tripeptide involved in the maintenance of the cellular redox stare and know to chelate copper and other metals was also not able to mobilize the AMBD bound Cu(I), reaction 10.

Discussion

The roles of all of the components in the various forms of the *cop* operon are not completely understood. All *cop* type operons contain a copper sensing CopY repressor and a copper ATPase, usually of the CopA type. CopB and CopZ are not as widely distributed. CopB is positioned to export Cu(I). Clearly, CopZ is capable of presenting Cu(I) to CopY but since CopZ is not present in all *cop* operons, CopY must be able receive Cu(I) by other means. CopA, which has a cytosolic domain homologous to CopZ, is cast as a Cu(I) importer but like all ATPase can also translocate Cu(I) out of the cell. The goal of these experiments was to look at the ability of the CopA metal binding domain to exchange Cu(I) with CopZ and CopY and, thereby, further dissect its function in the *E. hirae* copper homeostasis pathway.

Clearly the copper binding characteristics of the metal binding domain of CopA, AMBD, are very similar to those of CopZ with which it is highly homologous. Both CopZ and AMBD have a characteristic Cys-x-x-Cys binding. As copper is titrated into AMBD-MBP fusion it exhibits an increase in the copper-thiolate charge transfer band that plateaus at a stoichiometery of 1:1, (figure 3). Copper loading of this type is precisely what was discovered for CopZ [11], *B. subtilis* CopZ [18], and the second metal binding domain of the Menkes protein [19]. Intriguingly, Cu(I) bound to the metal binding domain of CopA in the maltose binding protein fusion, AMBD-MBP, transfers its Cu(I) to CopY (figure 3). The transfer was monitored through the titration of increasing amounts of Cu(I)AMBD-MBP into Zn(II)CopY (figure 3). The luminescence data indicates a ratio of two molar equivalents of per molar equivalent of Zn(II)CopY. The results are identical to those seen when Cu(I)CopZ is the titrant [11]. A homology model of the CopA metal binding based on the N-terminal domain of *B. subtilis* Cu(I) loaded CopA ATPase (PDB:1KQK) suggests it will have a surface that was very similar to that of CopZ (data not shown) [20]. AMBD and CopZ share 57.5% identity, the sequences are especially similar around the metal binding sites. The distribution of charges on the surface of CopZ has been shown to be important to the delivery of Cu(I) to CopY as their removal blocks the transfer [12]. Furthermore, addition of lysines to a homologous domain from the human Menkes Cu(I) ATPAse that was unable to transfer Cu(I) to CopY enabled the Menkes mutant to now function as a Cu(I) transporter [12]. Based on the Cu(I) exchange data, their sequence identity, as well as the predicted

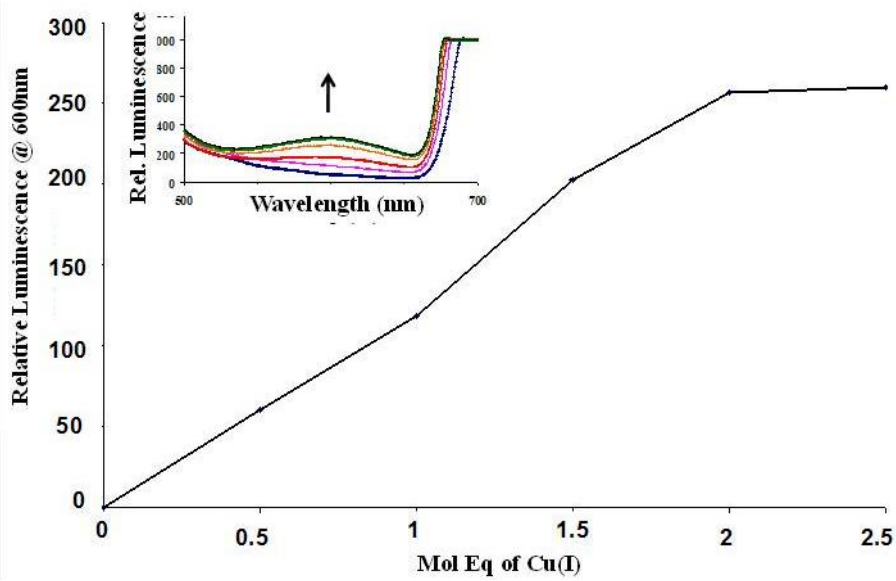

Figure 3. Luminescence titration from Cu(I) AMBD-MBP to Zn(II)CopY. **Main Figure**: Zn(II)CopY titrated with Cu(I) AMBD-MBP monitoring Cu(I)-S Luminescence- the luminescence spectra of the direct titration of Cu(I) AMBD-MBP into 5 nmol of Zn(II)CopY. Solvent- shielded Cu(I)-S bonds are luminescent at 600nm when excited at 295nm; 0.5 molar Eq of Cu(I) AMBD-MBP were titrated into the non-luminescent Zn(II)CopY. **Inset**: The luminescence at 600nm as Cu(I)AMBD-MBP is added increases to a molar Eq. of 2.0 Cu(I) AMBD-MBP per Zn(II)CopY before leveling off at 2.0 molar Eq.

structural similarity it seems plausible that both of these proteins could bind to CopY in a similar manner and transfer the Cu(I). It has been argued that in systems like *E. coli* that do not have a CopZ type chaperone that a chaperone could be formed by a specific proteolysis of its soluble N-terminal domain from its Cu(I) pump [4, 21, 22].

In addition to supporting the transfer of Cu(I) from AMBD to CopY the ultrafiltration assays also support the Cu(I) between AMBD and CopZ (table 1). The reverse Cu(I) transfer from CopZ to CopA, as part of the detoxification process, was proposed by Banci *et al* based on protein-protein interactions detected by NMR for the N^{15} Cu(I) labeled experiment in which Cu(I) transfer was tracked in the bacteria *B. subtilis* [18]. Similarly, *A. fulgidus* CopA metal binding domain have been suggested to transfer copper to that organism's CopZ, the CopA ATPase [23].

With this in mind, Cu(I) transfer was tested (table 1) in *E. hirae* from CopZ to AMBD (reverse transfer) and from CopZ to AMBD (forward transfer). The understanding of the Cu(I) transfer pathway in *E. hirae* between AMBD, CopZ and CopY will provide insight into the pathways of more complex organisms.

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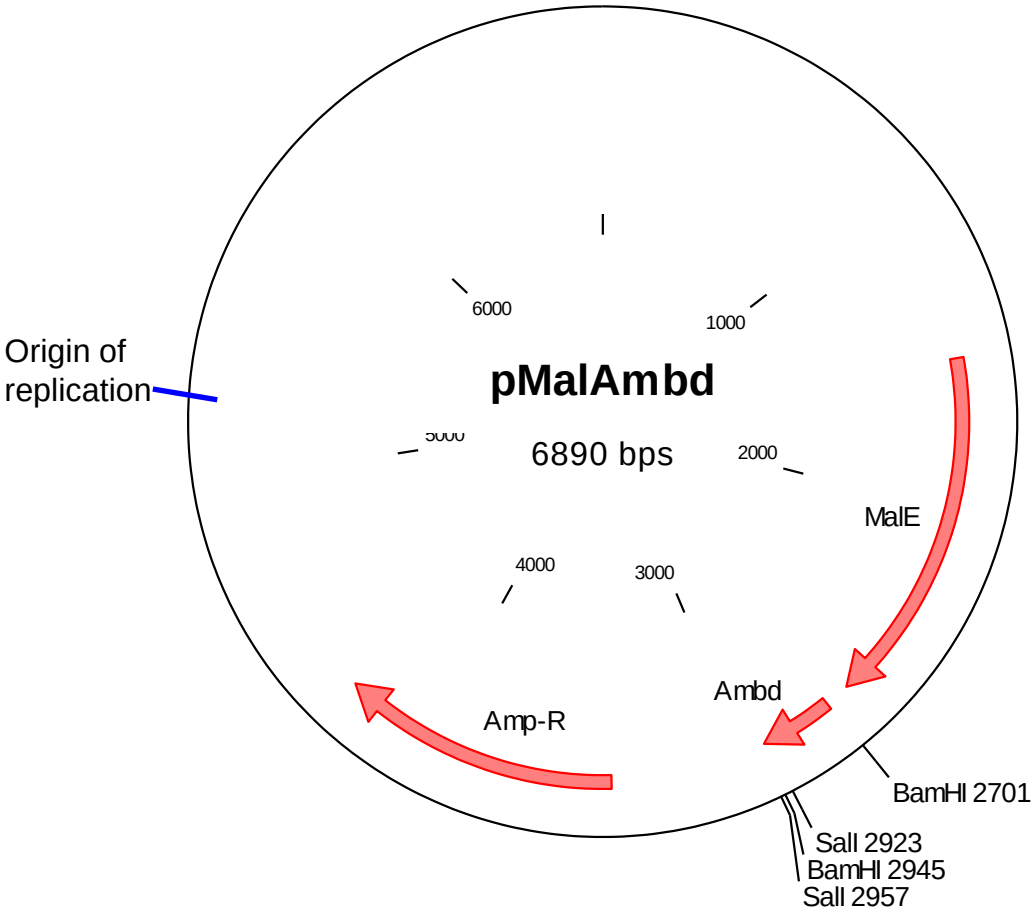
Supplemental information



Plasmid, pAMBD-MBPmbd, encodes the cytoplasmic portion of the CopA protein fused to the maltose binding protein (AMBD-MBP).